

Use of a Simple Fabrication Process to Produce a Biosensor: The 3-Hydroxybutyrate Dehydrogenase Case

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Abstract: This study was aimed to construct a biodegradable but reliable 3- β -hydroxybutyrate biosensor. In this context a versatile paper based biosensor, quickly, easily and cheaply fabricated is reported. The procedure of fabrication is based on the assumption that the introduction of the enzyme in the carbon ink will allow enzyme stabilization and facilitate the study of the catalysis of enzymes and the detection of substrates. To prove this concept we use the enzyme 3-hydroxybutyrate dehydrogenase, in aqueous solution. This enzyme was chosen because it catalyzes the 3- β -hydroxybutyrate, which results from ketoacidosis. The quantification this substance in the diabetics' blood is very important as it can increase the reliability of the diagnosis of glycaemia. To prove the multi-use of this biosensor we not only study the redox process in steady state and during the catalytic process, but also detected and quantify the 3- β -hydroxybutyrate. Our results showed that it was possible to study the redox process that occurred during the catalysis and to confirm the amino acid residues that participate in it. It was also observed that glucose and ascorbic acid can interfere in the detection and quantification of the 3- β -hydroxybutyrate, what should be in mind when the quantification of the 3- β -hydroxybutyrate is made in blood samples.

Key words: Diabetics, paper biosensor, screen-printing, 3- β -hydroxybutyrate dehydrogenase.

1. Introduction

The diabetic population increased and the perception of the consequence that it can cause not only to the individual (diseases, death, and inability to work) but also to the society (health care, economic impact on health system) lead to the necessity of its control [1-3]. The production of biosensors that can be cheap, easily made, reproducible and reliable are a goal that will allow the universalization of sensor coupled POC (point-of-care) diagnostic devices.

The use of glucose sensors coupled POC diagnostic devices, also known as glucometers, is one of the most used methods nowadays to measure the blood glucose concentration. Although it is simple, quick and an easy way to measure the glucose concentration, and monitor glucose levels, it is expensive since usually diabetics should know this information daily, and also is not free

of risks [3-10]. In diabetic patients, the ketoacidosis analysis is essential to prevent wrong diagnostics in diabetics' type 1. In healthy individuals, normal amounts of 3- β -hydroxybutyrate are below 1 mM, in individuals with hyperketonaemia this value can range from 1 mM to 3 mM [11-12].

The rapid determination of 3- β -hydroxybutyrate in blood is crucial to a quick and correct diagnosis, but only few 3- β -hydroxybutyrate biosensors have been reported and some of them are screen-printing electrodes [11, 13, 14]. In some of these sensors, lost in enzyme activity is reported, and attributed to mediator inhibition. They also report interferences with other electro active species in solution.

Some of these amperometric biosensors are based on redox mediators' reactions [15, 16]. The mediators used were 1,10-phenanthroline quinine and potassium ferricyanide. However, none of these systems detects 3- β -hydroxybutyrate directly, increasing the system complexity and cost.

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There are many advantages of development biosensors that can directly sense the presence of 3- β -hydroxybutyrate, using a simple enzymatic system 3-Hydroxybutyrate Dehydrogenase (HBH). In this context we developed a simple biosensor that, without modification of the surface working electrode, makes possible the detection of 3- β -hydroxybutyrate.

HBH is an oxidoreductase that presents a tetrameric structure. In each subunit of the tetrameric enzyme, the substrate (D-3-hydroxybutyrate) is trapped on the nicotinamide plane of the bound NAD^+ , and it has bounds to the amino acid chain [17] (Fig. 1).

The methyl group of the NAD^+ is accommodated in the nearby hydrophobic pocket, which makes possible a hydrogen bond from the OH group of the substrate to the hydroxyl group of Tyr155 at the active center. A comparison of the protein structure with and without ligands indicates that the Gln196 residue participates in the formation of additional hydrogen bonds. It is likely

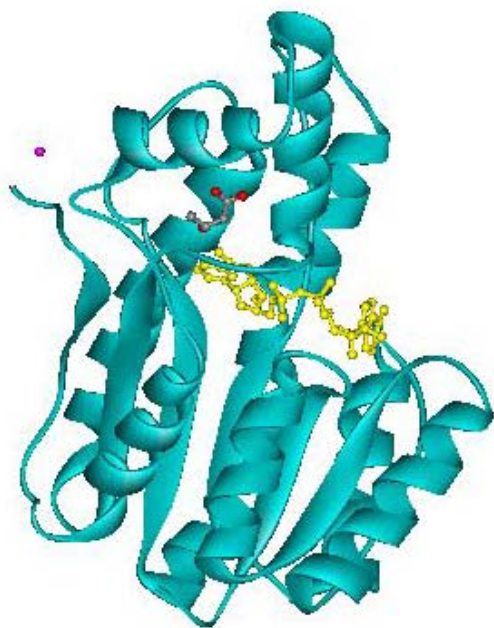


Fig. 1 PDB crystal structure of a tetramer unit of β -3-hydroxybutyrate dehydrogenase from *Alcaligenes faecalis*, complexed with NAD^+ (in the middle in yellow) an Na atom in the top (purple) near the amino acid chain (light blue) and a substrate β -3-hydroxybutyrate (gray and red) (PDB code: 5B4T) [16]. Figure prepared with Viewerlite program.

that this situation can facilitate H-atom movements as the trigger of the catalytic reaction [17].

The redox system NADH/NAD^+ is very important in nature since it is a cofactor of a large number of enzymes, but also because it is important in biofuel cells and bioreactors [18-20]. However, it is also a challenge to develop biosensors, once there is the possibility of NAD^+ freely lives the enzyme, becoming the enzyme in an inactive conformation, but also the formation of inactive products (NAD_2 dimer). Another problem is that the oxidation of NADH is usually irreversible, it occurs at high potential, NAD^+ act as an inhibitor of the direct electrode process or even provokes electrode surface poisoning [21-24]. To overcome these difficulties, efforts have been made using different strategies as surface modification, use of mediators [13, 16, 25-27] or use new materials (carbon nanofibers, carbon nanotubes, graphene and composites) as immobilization matrix for biomolecules [18, 19, 22, 28-34]. These paper devices are screen-printing electrodes that have an important role in the biosensing development, once they have numerous applications in biochemical fields [35-38]. The versatility of these devices, which can sense diverse substances (from metals to enzymes, pesticides, antigens, biomolecules) and are easily made, give a fundamental place in the electrochemical research nowadays.

We used the system HBH/NAD^+ as the key base to develop a new biosensor [39, 40]. The electron transfer occurs between the redox active enzyme center and the electrode surface and allows the use of the redox couple NADH/NAD^+ as a reliable source of information related to the detection of 3- β -hydroxybutyrate with a β -3-hydroxybutyrate dehydrogenase/ NAD^+ biosensors.

2. Experimental

2.1 Materials

All reagents used were of analytical grade. 3-hydroxybutyrate dehydrogenase (HBH) from

Rhodobacter sphaeroids (aqueous solution (2 mL), 10 mg), was used with no further purification, 3- β -hydroxybutyrate, β -nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH), potassium chloride and potassium ferrocyanide were acquired from Sigma-Aldrich. All buffers used in this work were commercial and purchased from ROTH (Germany). The electrolyte was a buffer solution with potassium chloride (0.1 M). All solutions were prepared with electrolyte.

2.2 Fabrication of the Biosensor

The carbon ink and Ag/AgCl ink were purchased from Conductive compounds. The ink base of the working electrode was the carbon ink in which was added enzyme.

A Xerox Color Qube 8570 printer from Xerox was used to print the hydrophobic region of the devices. The paper used was Whatman n.^o1 chromatographic paper, and the wax was obtained from Xerox. After the wax printing, the wax was heat treated during 10 s in a hot plate (150 °C). After that, the paper, cooled at room temperature and it was ready to perform the screen-printing technique. The configuration system designed was a three electrode system with an Ag/AgCl reference electrode, a carbon counter electrode and a working electrode based in carbon ink. The conductive circuit was screen-printed with silver ink, which was deposited above the hydrophobic matrix (wax). Then the mesh was removed and the device was allowed to heat at hot plate (60 °C) during 8 minutes. Once the construction of the conductive circuit was concluded, the counter electrode was printed and dried the same way as described before. The other two electrodes had the same screen-printing treatment.

2.3 Working Electrode Preparation

The enzyme, in aqueous solution, and cofactor, in the solid state, were mixed with the carbon ink. This mixture was used to do the working electrode.

2.4 Electrochemical Detection

During the electrochemical measurements, a drop of the interest solutions (2 μ L) is spotted in the hydrophobic channel between the wax-limited zones and dispersed through the paper matrix in a few seconds, being in contact with the three electrodes. The electrochemical behavior of enzyme was experimentally characterized through cyclic voltammetry.

All electrochemical acquisitions and measurements were performed in a Gamry ESA419 data acquisition system, using PHE 200 physical electrochemical and PV 220 physical electrochemical software coupled with a Gamry instruments (reference 600) potentiostat/galvanostat (ZRA) and the data analysis were processed by Gamry software package. All the experimental procedure was performed in normal atmosphere in the presence of oxygen.

3. Results and Discussion

The major goal of this research was to fabricate a 3- β -hydroxybutyrate biosensor that did not need a mediator, as in the previous biosensors reported, which not only would decrease the price of the apparatus but also nullifies its influence in the biosensor detection-mediator inhibition. The simplification and the reduction of time-consuming in the biosensor manufacture, in comparison to previous ones, were another aim of this research (see 2.2 Fabrication of the biosensor). To ensure the enzyme activity—one of the problems reported in earlier research—the strategy was to introduce the enzyme as it was purchased and its cofactor NADH, in the working electrode mixture. The results obtained are described below.

3.1 Electrochemistry of HBH Using a Screen-Printing Electrode

With the aim of developing a 3- β -hydroxybutyrate (HB) biosensor, a metabolite produced by the human body, we developed a screen printing biosensor that can sense its presence. To achieve this objective, we fabricated a working electrode using a mixture of

NADH (35 mg) and HBH (0.25 mg) in carbon ink (814 mg). The addition of the cofactor NADH was to ensure the enzyme activity.

The cyclic voltammograms were recorded at room temperature (22 °C), at sweep rate between 20-100 mVs⁻¹. The direct electron transfer between the redox active center and the electrode surface occurred at low potentials (Fig. 2).

Two well-defined anodic and cathodic peak currents appeared in the voltamogramas. To ensure the repeatability of the results, more six experiments were conducted in the same conditions using six different screen printing electrodes.

Using the data of the seven experiments, using seven different biosensors, it was possible to obtain the following results: a well-defined cathodic peak appears at $E_p^a = 81 \pm 6$ mV and anodic peaks, were observed at $E_p^c = -134 \pm 27$ mV. A peak-to-peak separation, ΔE_p , with a value of (232 ± 14) mV vs Ag/AgCl ($n = 7$) was obtained and a formal potential of $E^{0'} = (-26.5 \pm 10)$ mV vs. Ag/AgCl ($n = 7$) was calculated. A ratio of anodic to cathodic peaks current, $|i_p^a/i_p^c|$ was also

estimated near the unit. Although E_p and $i_p/v^{1/2}$ are independent of the scan rate, $|i_p^a/i_p^c|$ is near the unit, peak-to-peak variation, ΔE_p , is far from the theoretical value ($59/n$ (mV), n = electron number). The parameters obtained in these experiences reflect a quasi-reversible redox process, controlled by diffusion [40-42].

3.2 Catalysis of 3- β -Hydroxybutyrate by HBH Using a Screen-Printing Electrode

Cyclic voltammetry was used to analyze the behavior of HBH in the presence of increasing concentrations of HB. The direct catalysis of 3-hydroxybutyrate dehydrogenase (HBH) activated (HBH with cofactor—NADH) in pH 7 was observed. The voltammetric data obtained in these conditions at the screen printing electrodes were assigned to the redox process involving the NADH and the HBH catalytic center. An increase in the cathodic peak current was observed during 3- β -hydroxybutyrate additions. The calibration curve in the presence of different concentrations of HB is shown in Fig. 3.

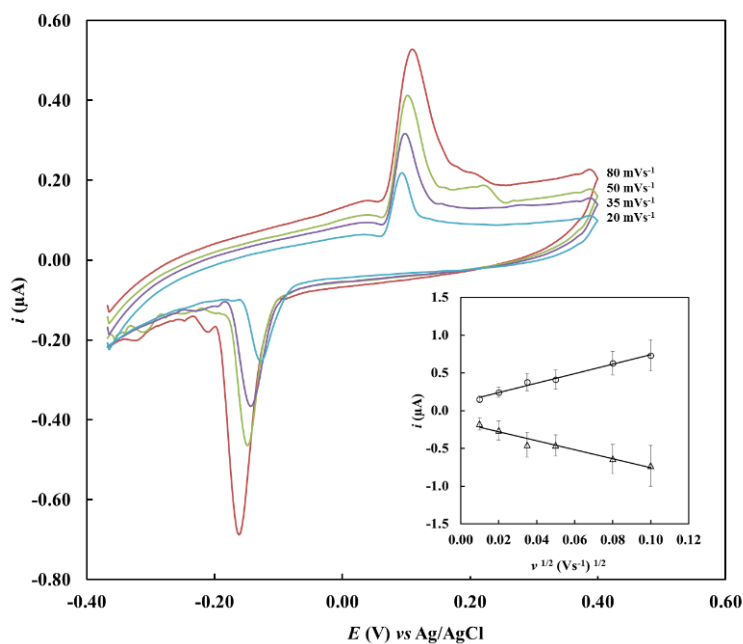


Fig. 2 Cyclic voltammograms at a screen at a screen printing electrode with a three electrode system configuration with an Ag/AgCl reference electrode, a carbon counter electrode and a working electrode with NADH (35 mg) and HBH (0.25 mg) in carbon ink (814 mg) in 2 μL of PBS (7.0) with KCl (0.1M). Insertion: Variation of the anodic and cathodic peak current with the square root of the sweep rate. (\circ): $i_p^a = 2.627v^{1/2} + 0.118 \mu\text{A}$, $R = 0.99$, $n = 7$; (Δ): $i_p^c = -2.592 v^{1/2} + 0.076 \mu\text{A}$, $R = 0.99$, $n = 7$).

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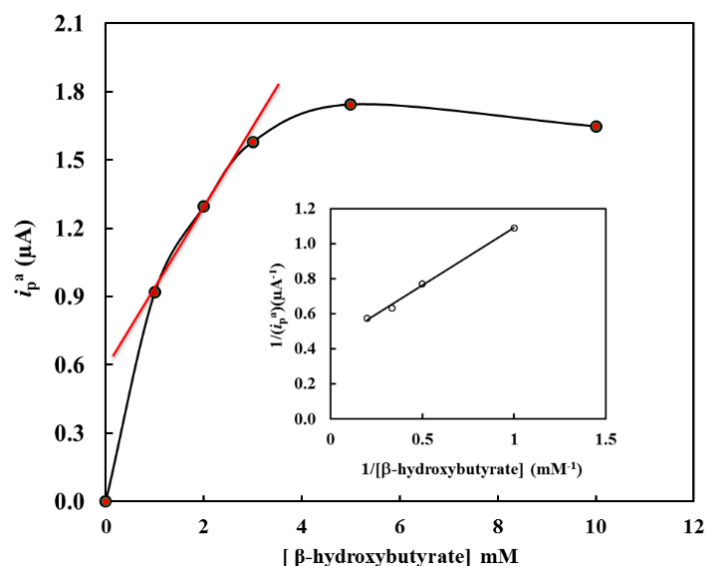
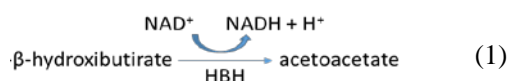


Fig. 3 Variation in the anodic catalytic current of HBH with 3- β -hydroxybutyrate concentration detected at screen printing electrode with a three electrode system configuration with an Ag/AgCl reference electrode, a carbon counter electrode and a working electrode with NADH and HBH in carbon ink. The voltammograms were recorded in 2 μ L of PBS (7.0) with KCl (0.1 M) with different 3- β -hydroxybutyrate concentration. Insertion: Michaelis-Menten Lineweaver-Burk linearization: $1/i_p^a = 0.6561/[3\text{-}\beta\text{-hydroxybutyrate}] + 0.4332$, $R^2 = 0.9965$, $K_m = 1.54$ mM, $I_{cat}^{max} = 2.3$ mM. The best curve fit to Michaelis-Menten kinetics (—); Linear variation of anodic catalytic current between 1 and 3 mM of 3- β -hydroxybutyrate was $i_p^a = 0.330 [3\text{-}\beta\text{-hydroxybutyrate}] + 0.603$, $R^2 = 0.9936$, $n = 3$ (three measurements) for each point (—).

The addition of 3- β -hydroxybutyrate produced a slight change in the electrochemistry parameters to: $\Delta E_p = (239 \pm 15)$ mV vs. Ag/AgCl ($n = 13$), and the estimated $E^{0*} = (-22.0 \pm 9)$ mV vs. Ag/AgCl ($n = 13$). The formal potential, E^{0*} , is near the value obtained in buffer solution signifying that the redox groups responsible for the catalytic process and for oxidation and reduction in non-turnover conditions are the same.

The reaction that occurs in the catalytic center is the following one:



The curve was adjusted to Michaelis-Menten enzyme kinetics description and constant values were estimated from cyclic voltammetric experiments (Fig. 3). The K_m values were found to be (1.52 ± 0.03) mM. The values reported in the literature for a screen-printed iridium—modified working electrode was (2.26 ± 0.238) mM, at 25.8 $^\circ\text{C}$) [13] and with Single-walled carbon nanotubes—modified screen

printing electrode (SWCNT-modified SPCE) were 4.61 mM (pH 7.5) [14] and 0.41 mM at pH 8.5 (0–33 mM NAD) [42]. These data are near and in the same order of magnitude of the present. Although the K_m parameters can be used to evaluate the enzyme activity, it is possible to see that the K_m values diminish with the temperature and that at 37.5 $^\circ\text{C}$ its value is (1.83 ± 0.302) mM [13].

It is possible to use the linear fit between 1 mM and 3 mM of β -hydroxybutyrate to identify the ketone quantity present in blood samples, Fig. 3. This detection range is useful to perceive the ketonaemia and can be used for clinical measurements.

These electrodes were also used to examine the formal potential variation with the pH in the presence of 3- β -hydroxybutyrate (10 mM) (Fig. 4).

The formal potential in electrolytes is almost constant in all the pH range (Insertion), but after the addition of 3- β -hydroxybutyrate it varies (Fig. 4). From the experimental data it was possible to estimate $pK_{a1} = 4.0$, $pK_{a2} = 10.2$. These constants can be attributed

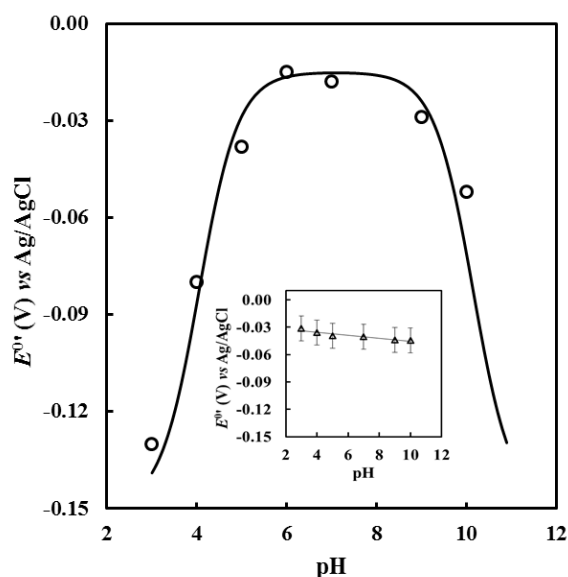


Fig. 4 Variation the formal potential with the pH. The best curve fit allowed the estimation of $pK_{a1} = 4.0$, $pK_{a2} = 10.2$. Measurements with different electrodes each point is the media of two measurements with different electrodes. Insertion: formal potential variation with the pH.

to the aspartic acid (pK_a 3.9) or glutamic acid (pK_a 4.0) and Tyrosine (10.46) respectively [43]. In fact, near the catalytic center there is one residue that has an important paper in the catalysis, Trp155. It is worthwhile to mention that in crystal structure the HB is the molecule that binds to the catalytic center (not acetoacetate), as well as NAD^+ (not $NADH$). Hydrophilic interactions are established in the complex $HBH-NAD^+-HB$ thought the bounded of HBH by the two carboxylate oxygen's through four hydrogen bonds. But HB is also accommodated in a hydrophobic pocket surrounded by Ala143, His144 and Trp187 e Trp257 [17]. So these experimental results are in agreement with the crystal data.

3.3 Influence of Interferentes Species in the Catalysis of HBH

The main goal of this project was to construct an electrode that could be used to analyze diabetic's blood. So it was necessary to examine the interference of glucose and ascorbic acid usually present in blood. The rapid detection of these three compounds (glucose,

ascorbic acid and 3- β -hydroxybutyrate) in serum and blood may permit a reliable diagnose not only of glycaemia but also of ketonaemia. In order to analyze the specificity of this sensor, the formal potential signal variation with glucose was studied. In amounts between 5-150 mM the glucose presence did not implicate a change in formal potential, $E^{0'} = (-32 \pm 5)$ mV vs. Ag/AgCl. These findings indicate that the redox center where glucose is being detected is also the catalytic center. The catalytic currents i_p^{cat} , calculated as the rate i_p^a after/ i_p^a before glucose addition, had an increase in low concentration, but to values higher than 25 mM there was a decrease, and after 50 mM the i_p^{cat} values do not change with glucose addition (Fig. 5).

Nevertheless, in healthy people the glucose range is from 4.4 mM to 6.6 mM [3]. So, to use this electrode in real samples we must do careful analysis of the results because glucose may compete with HB, increasing artificially the electrochemical signal which may be read as an increase in HB amounts.

The presence of ascorbic acid was also studied. The electrochemical signal variation, before and after the addition of ascorbic acid was analyzed. In quantities in the range of 28 μ M to 2.8 mM the catalytic current (rate i_p^a after/ i_p^a before the addition) was 1.36 ± 0.09 . However, for concentrations higher than 2.8 mM a

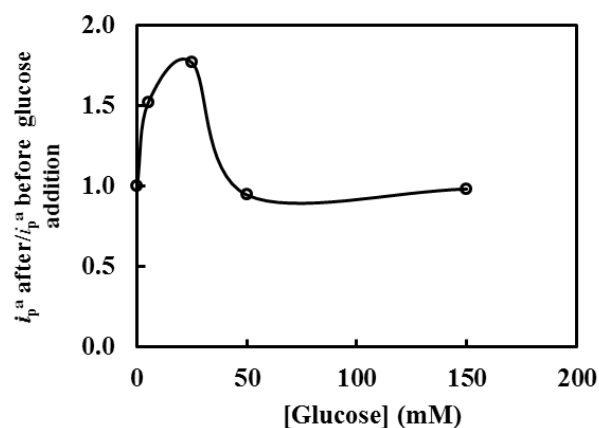


Fig. 5 Variation of i_p^{cat} (i_p^a after/ i_p^a before glucose addition) with glucose concentration. The cyclic voltammograms were recorded with a scan rate 100 mVs^{-1} in the electrolyte (PBS Buffer (pH 7) with KCl (0.1 M)), in the presence of HB (10 mM).

greater increase in catalytic current peaks is observed. It is interesting to highlight that the maximum quantity of ascorbic acid in plasma in a recent study was reported to be 100 $\mu\text{mol/L}$ [44], and the reference range in the blood is between 34 $\mu\text{mol/L}$ and 113 $\mu\text{mol/L}$. So with this in mind, and the slight increase observed in the catalytic current for concentrations below 2.8 mM, it's necessary to look at the results carefully when a clinical diagnosis is done. It is clear from our data that not only the presence glucose can induce in mistaken diagnoses of hyperketonaemia but also the ascorbic acid.

4. Conclusions

In this work we used a simple but efficient procedure to immobilize the enzyme (3-hydroxybutyrate dehydrogenase) and the cofactor (NADH) in the matrix of the working electrode. We use the cofactor NADH to ensure the enzyme activity. This method does not need any drastic treatment, so the enzyme maintains the native conformation without denaturation. This fabrication proceeding also allowed not only the detection and quantifications of the 3- β -hydroxybutyrate in the range of interest 1-3 mM, but also the catalysis study.

It was possible to verify that the catalytic center was also the redox center in non-turnover conditions, and we also could identify the amino acid responsible for that (Trp). This attribution was based on experimental data that corroborate the crystallographic data's publish recently.

It's important to highlight that this research is driven by the need of a more reliable diagnosis of diabetes, and the development of biodegradable, cheap but reliable 3- β -hydroxybutyrate biosensors. In future research, this biosensor should be tested with human blood, to evaluate its efficacy, reliability and usefulness in *in vivo* situations.

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